PREPARATION OF TOXIC RADIO-IODINATED RICIN FOR USE IN RICIN–RIBOSOME INTERACTION STUDIES

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1. Introduction

Radioactive labelling of protein is useful to study interactions and binding of these molecules to cells, membranes as well as to molecular structures. Lactoperoxidase-catalysed iodination of proteins has the advantage of high specific activity and simple methods of preparation. Moreover, the radioactivity is easy to detect. The method of Pagès et al. [1] can be well-controlled; under mild conditions tyrosyl residues are iodinated but not histidyl residues.

Ricin is a highly toxic protein interacting with the 80 S ribosomes [2–5] but its specific target, ribosomal protein or RNA, is not yet known. Radioactive ricin is useful to study this interaction. Inactivation of ricin as a function of the number of iodine atoms incorporated per molecule was studied. Conditions giving an iodinated-ricin of high specific radioactivity without loss of activity were determined.

2. Materials and methods

2.1. *Materials*

The seeds of *Ricinus communis* L. were kindly supplied by Société Française du Ricin, Marseille (France) and were imported from Paraguay.

Carrier-free Na ^{125}I (10 mCi/μg) was obtained from the Radiochemical Center, Amersham (Searle). Lactoperoxidase (EC 1.11.1.7) was purchased from Calbiochem and Sephadex G-25 came from Pharmacia, Uppsala.

ATP, GTP, creatine phosphate, creatine phosphokinase, non-labelled amino acids and hemin were from Sigma. L-[^{14}C]valine (140 mCi/mmol) was obtained from C.E.A. (Saclay). Omnifluor was from Nen Chemicals and N.C.S. solubilizer came from Amersham. All other chemicals were of the purest grade available from Boehringer (Mannheim) or Merck (Darmstadt). Adult albino male mice (OF1, E.S.P.O., Les Oncins, France) weighing 20 ± 2 g were utilized to study the toxicity.

2.2. *Preparation of ricin*

Ricin extracted from shelled seeds of *Ricinus communis* L. was purified to homogeneity as described earlier [6], concentrated to 1.65 mg/ml, and dialyzed extensively against 20 mM phosphate buffer, pH 6.8 (NaH_2PO_4, K_2HPO_4). This preparation, which is homogeneous in polyacrylamide gel electrophoresis at different pH's, has no hemagglutinating effect, no proteolytic action and is free of mitogenic activity on human peripheral lymphocytes [7,8]. It is chemically and biologically different from castor bean agglutinins or lectins [6–8] isolated by Tomita et al. [9], Nicolson and Blaustein [10] and Olsnes et al. [11].

2.3. *Protein determination*

Proteins were determined according to Lowry et al. [12] using human serum albumin as a standard.

2.4. *Determination of iodine ratio per mole of ricin*

The number of iodine atoms per mole of ricin was determined from the specific radioactivity of iodine and the molar concentration of ricin (mol. wt., 66 600 [7]). Radioactivity was measured from an aliquot of the incubation mixture by counting in a

Packard auto-gamma scintillation spectrometer. Samples of Na ^{125}I with a specific radioactivity of 10^9 cpm/μmol were used.

2.5. *Analytical Sephadex G-25 chromatography*

The reaction mixture was promptly loaded on a Sephadex G25 column (1.0 × 30 cm) previously equilibrated with 0.25 M sodium acetate buffer, pH 5.8. The same buffer was used for elution. Fractions of 1.7 ml were collected and counted. All operations were carried out at 4°C.

2.6. *Eukaryotic cell-free protein synthesis*

A cell-free globin synthesizing system was prepared from rabbit reticulocytes according to the method of Lockard and Lingrel [13], as modified by Rhoads et al. [14]. The incubation mixture (1 ml) contained: 0.40 ml of reticulocyte lysate, 75 mM KCl, 2 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 40 units of creatine phosphokinase, 15 mM hemin, 2 μCi of L-[^{14}C]valine (140 mCi/mmol) and 0.1 mM of each of the other 19 non-radioactive amino acids. The incubation was carried out at 27°C with different radio-iodinated ricin preparations. After 10, 20, 30, 60 and 90 min, 50 μl samples were pipetted onto Whatman 3 MM paper discs and treated as described by Mans and Novelli [15]. Each disc was placed in a counting vial and shaken with 0.50 ml of NCS solubilizer at 37°C for 2 h. Radioactivity incorporated into proteins was determined by counting in a liquid scintillation spectrometer (Beckman LS-250) using 10 ml of toluene–Omnifluor (4 g/l). Similar experiments were carried out with pure native non-radioactive ricin, or without ricin.

2.7. *Toxicity*

For rapid toxicity studies, solutions of iodinated-ricin preparations (1 μg in 0.10 ml of saline) were injected intraperitoneally into mice. For each dose, 10 mice were injected and the same number of animals receiving 0.10 ml saline served as the control set. The mice were observed for 24 h and mortality was noted. The toxicity was evaluated by the number of mice which died divided by the total number of experimental animals. Post mortem examinations were also made to avoid any misinterpretation. Pure native ricin, at a dose of 1 μg/mouse, killed all the animals under study after 24 h [7].

2.8. *Isolation of ribosomes and their subunits from rat liver*

The ribosomes 80 S and their subunits 60 S and 40 S, isolated according to the procedures described by Blobel and Sabatini [16,17], were dialyzed against 50 mM triethanolamine buffer, pH 7.4, 5 mM $MgCl_2$, 25 mM KCl, 20 mM 2-mercaptoethanol. After incubation with radio-iodinated ricin, the 80 S ribosomes and the 60 S and 40 S subunits were separated from unbound ricin by 5–20% sucrose gradient centrifugation and the collected fractions were counted.

3. Results

3.1. *Radio-iodinated ricin*

Ricin was iodinated with Na ^{125}I according to the procedure of Pagès et al. [1] with slight modifications. The iodination mixture (1–3.0 ml) was prepared so that the molar ratio of iodide to toxin varied from 1 to 40. A ten-fold molar excess of H_2O_2 with respect

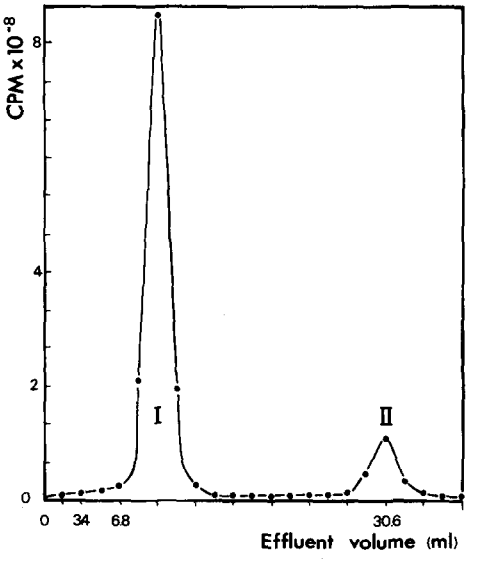

Fig.1. Separation of monoiodinated-ricin from free iodide on a Sephadex G25 column (conditions described in Material and methods). The reaction mixture contained 4.95 mg ricin, $150 \cdot 10^8$ cpm Na ^{125}I, 9 μl H_2O_2 100 mM, 70 μg lactoperoxidase. I: Monoiodinated-ricin; II: Free iodide.

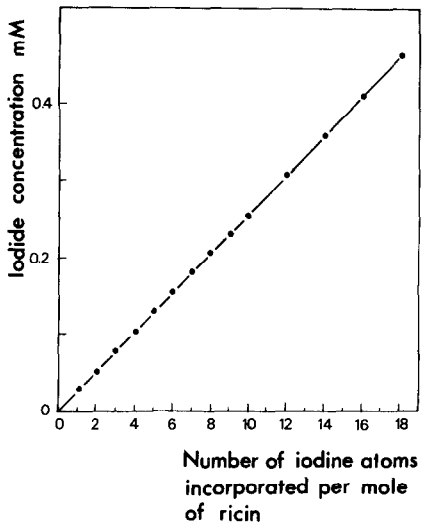

Fig.2. Iodine atoms incorporated per mole of ricin as a function of the iodide concentration.

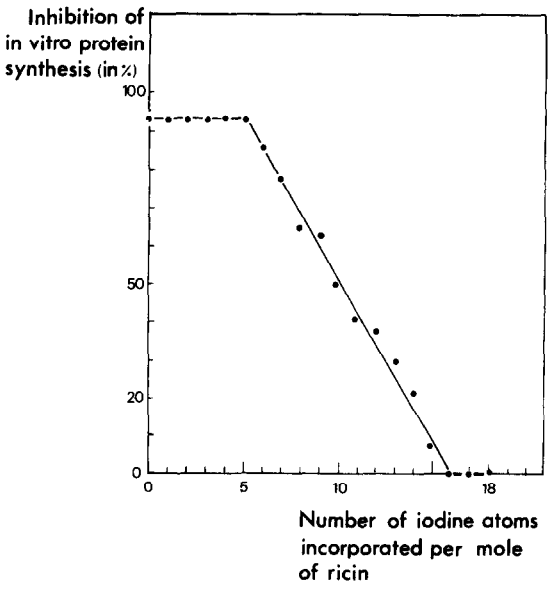

Fig.3. Influence of iodination level on protein synthesis in a rabbit reticulocyte cell-free system.

of iodide was used. The reaction was catalyzed by 5–75 μg of lactoperoxidase. Aliquots of H_2O_2 were added at 1 minute intervals with constant stirring. After 15 minutes, the excess H_2O_2 was destroyed with 5.5 mM L-cystein. The reaction mixture was immediately loaded on a Sephadex G-25 column at 4°C. Washing with 0.25 M sodium acetate buffer separates iodinated ricin from the unbound iodide (fig.1).

3.2. *Number of iodine atoms per mole of ricin*

In the preceding experiments, the number of iodine atoms incorporated per molecule ricin was varied by changing the concentrations of iodide. Fig.2 shows the number of iodine atoms bound per mole of ricin versus the iodide concentration in the reaction mixture.

3.3. *Inhibition of protein synthesis by different iodinated-ricin molecules*

Each radio-iodinated ricin preparation was tested for protein synthesis inhibition on a reticulocyte lysate. Kinetics of protein synthesis show that ricin retains its inhibitory activity when 1–5 iodine atoms are bound per molecule. At higher iodination levels its inhibitory effect decreases (fig.3).

3.4. *Toxicity of the different labelled ricin molecules on mice*

As shown in Table 1, ricin was labelled with 1–5 iodine atoms per molecule without loss of toxicity. Above five ^{125}I per molecule of toxin, the toxicity decreases with increased iodination.

3.5. *Action of fully active ^{125}I-ricin on rat ribosomes*

Ricin labelled with two iodine atoms per molecule

Table 1
Toxicity of different radio-iodinated ricin molecules on mice

Number of iodine atoms per molecule of labelled ricin	Toxicity
0	10/10
1	10/10
5	10/10
10	6/10
12	4/10
14	2/10
18	0/10

Doses of 1 μg of each radio-iodinated ricin preparation in 0.10 ml saline were injected into 10 mice. Toxicity was estimated by the number of mice which died within 24 h divided by the total number of experimental animals.

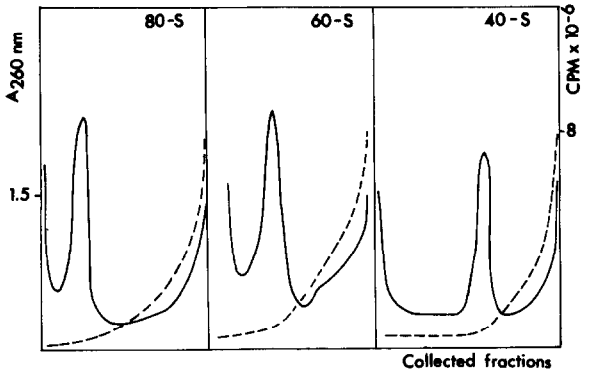

Fig.4. Study of action of radio-iodinated ricin on 80 S, 60 S and 40 S rat liver ribosomal particles. After incubation with labelled ricin the ribosomal particles were separated from unbound ricin by 5–20% sucrose gradient centrifugation. Absorbance at 260 nm (————) and radioactivity (- - - - - - - - -) were estimated on the collected fractions.

was incubated with ribosomes or their subunits and centrifuged on a sucrose gradient. Iodinated ricin is not bound to 80 S ribosomes (fig.4) nor to 60 S and 40 S subunits. It is to be noted that the same preparation of radio-iodinated ricin inhibits protein synthesis in a reticulocyte lysate to the same extent in the presence of 0.5 M sucrose or 0.3 M lactose in the reaction mixture.

4. Discussion

The method described permits the preparation of radio-iodinated ricin containing 1–18 iodine atoms per molecule of toxin. Radio-iodinated ricin does not lose its inhibitory activity on protein synthesis in vitro. Its toxicity in vivo is retained when less than 6 iodine atoms are incorporated per ricin molecule. When more than five iodine atoms are incorporated, both the inhibition of protein synthesis and the toxicity on animals decreases progressively. When 10 tyrosyl residues of ricin are iodinated, there is a 50% loss of activity. When all 18 tyrosyl residues of ricin [7] are iodinated, toxicity is completely lost. This suggests that these tyrosyl residues play a role in the activity of the toxin, as is the case for some proteins, especially antibodies [18,19].

The method employed is milder than the other iodination methods such as the triiodide ^{131}I technique (done at pH 9.0–9.5, [20]) or the chloramine T method [21] and iodinated molecules of high specific radioactivity ($5 \cdot 10^3$ cpm/ng of mono-iodinated ricin) are easy to obtain. The iodination technique is widely applicable and could be used to prepare radio-iodinated A- and B-chains of ricin [22] as well as the toxic tryptic ricin peptides [23] which would facilitate the study of ricin's toxicity and its action on protein synthesis.

Since Dirheimer et al. [24] discovered ricin's inhibition of protein synthesis on the level of rat ribosomes, Montanaro et al. [2] and Sperti et al. [3] have shown that the target of action of ricin on protein synthesis is the 80 S ribosome and particularly the 60 S ribosomal subunit. This point was later reinvestigated by Carrasco et al. [5] and Olsnes et al. [25]. It was therefore interesting to see if radio-iodinated ricin of high specific activity could be bound to these ribosomal structures. After incubation with radio-iodinated ricin, rat liver 80 S ribosomes and their 60 S and 40 S subunits are reisolated on sucrose gradients. Ricin is not retained on any of the ribosomal particles.

It would therefore seem that the toxicity of ricin is not due to its binding to ribosomes, but rather to an enzymatic mechanism. This possibility was proposed by Le Breton and Moulé [26] and further investigated by Olsnes et al. [25].

However, like α-amanitin or bacterial toxins such as diphtheria toxin, the toxicity of ricin on animals does not seem to be due to the potent inhibition of protein synthesis. Since the experimental animals die very quickly (10 h with high doses of ricin), protein synthesis inhibition is an insufficient explanation of the ricin's high toxicity. The mechanism of toxicity therefore remains to be elucidated.

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